

Extended differentiation of veins and stomata is essential for the expansion of large leaves in *Rheum rhabarbarum*

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PREMISE OF THE STUDY: The densities of veins and stomata govern leaf water supply and gas exchange. They are coordinated to avoid overproduction of either veins or stomata. In many species, where leaf area is greater at low light, this coordination is primarily achieved through differential cell expansion, resulting in lower stomatal and vein density in larger leaves. This mechanism would, however, create highly inefficient leaves in species in which leaf area is greater at high light. Here we investigate the role of cell expansion and differentiation as regulators of vein and stomatal density in *Rheum rhabarbarum*, which produces large leaves under high light.

METHODS: *Rheum rhabarbarum* plants were grown under full sunlight and 7% of full sunlight. Leaf area, stomatal density, and vein density were measured from leaves harvested at different intervals.

KEY RESULTS: Leaves of *R. rhabarbarum* expanded at high light were six times larger than leaves expanded at low light, yet vein and stomatal densities were similar. In high light-expanded leaves, minor veins were continuously initiated as the leaves expanded, while an extended period of stomatal initiation, compared to leaves expanded at low light, occurred early in leaf development.

CONCLUSIONS: We demonstrate that *R. rhabarbarum* adjusts the initiation of stomata and minor veins at high light, allowing for the production of larger leaves uncoupled from lower vein and stomatal densities. We also present evidence for an independent control of vein and stomatal initiation, suggesting that this adjustment must involve some unknown developmental mechanism.

KEY WORDS cell size; epidermis; leaf expansion; light intensity; stomatal development; stomatal density; vein density.

Precise coordination between the demand for water at the evaporating surfaces in the leaf and the supply of water to those surfaces appears to be a universal feature of plant leaves. This coordination is usually achieved by balancing stomatal density, which largely determines demand (Franks and Beerling, 2009), with leaf vein density, which determines supply (Sack and Frole, 2006; Brodribb et al., 2007), through a mechanism that involves altering the densities of these two anatomical features through changes in cell size (Brodribb and Jordan, 2011; Carins Murphy et al., 2012). The developmental coordination between stomatal and vein density is driven by the selective pressure to optimize the benefit-to-cost ratio of leaf construction such that an investment in a leaf vein network is sufficient to maintain stomata supplied with the minimum amount of water required to allow them to open fully under saturating light conditions (Brodribb

and Jordan, 2011). In a number of developmental and hormone synthesis mutants, a loss of coordination of vein and stomatal density results in reduced maximum leaf conductance and gas exchange (Dow and Bergmann, 2014; Dow et al., 2014; McAdam et al., 2017).

Evidence for the coordination between vein and stomatal density has been found across and within species in response to varying environmental conditions, such as light intensity and humidity (Brodribb and Jordan, 2011; Carins Murphy et al., 2012, 2016, 2017; Zhao et al., 2017). A chief mechanism that induces these developmental changes in vein and stomatal densities involves passive changes in cell size, especially epidermal pavement cells (Carins Murphy et al., 2012; Sack and Buckley, 2016; Carter et al., 2017). With all leaves initiating the same numbers of veins and stomata, when cell sizes change, the densities of veins and stomata change.

Low vein and stomatal densities can be found in large leaves in which cell expansion continues for a long period of development, whereas in leaves in which cell expansion is limited, leaf size is reduced and vein and stomatal densities are higher (Carins Murphy et al., 2012). This passive determination of vein and stomatal density via variation in cell size is believed to be the primary mechanism by which leaves coordinate vein and stomatal densities in response to changes in environmental condition in most woody and herbaceous angiosperms (Carins Murphy et al., 2012, 2016). Typically, leaves that expand under low light or low leaf-to-air vapor pressure difference (VPD) have much larger cells and consequently lower vein and stomatal densities than leaves that expand under high light or high VPD (Carins Murphy et al., 2012, 2016). This reduction in vein and stomatal densities has a direct effect on the photosynthetic and hydraulic capacity of leaves (Sack and Frole, 2006; Brodribb et al., 2007; Xiong et al., 2014; Scoffoni et al., 2016; Zhang et al., 2018).

The passive dilution model appears to work well for a wide range of species, especially species that have larger leaves in the shade compared with sun. For those species, passive dilution can lead to a lower vein and stomatal density in the shade, without the need to change cell numbers. A reduced investment in veins and stomata in shade-adapted leaves is an optimal economical investment, given the lower photosynthetic yields of shade-grown leaves. However, many herbaceous species have a poor tolerance of shade and produce substantially larger leaves in the sun (Gay and Hurd, 1975; Dengler, 1980; Kalve et al., 2014). In such species, passive dilution without the addition of new veins and stomata would lead to lower vein and stomatal density under the very conditions where high densities, which are essential for high rates of gas exchange, are most beneficial.

As an alternative to passive cell expansion, the role of vein and stomatal differentiation during leaf expansion remains an understudied mechanism for developmental coordination of veins and stomata, particularly in species with larger leaves in the sun. The developmental responses of veins and stomata in some fern species indicate that increased stomatal differentiation may indeed play a role in determining the stomatal density of leaves grown under different light intensities (Carins Murphy et al., 2017). In addition, unlike major veins, which are formed at very early stages of leaf expansion, minor vein differentiation appears to be more flexible in terms of developmental timing, being continuously differentiated until the final stages of leaf expansion (Koizumi et al., 2000; Jun et al., 2002; Sack et al., 2012; Sack and Scoffoni, 2013). Thus, a prolonged initiation of minor veins could potentially lead to a maintenance of minor vein densities, regardless of leaf area or cell size, in large leaves (Sack et al., 2012).

Here we hypothesized that the coordination of vein and stomatal densities in a shade-intolerant species with large leaves in high light environments requires the active differentiation of veins and stomata during leaf expansion. To test this hypothesis, we analyzed leaf anatomical traits during leaf expansion in the geophyte species *Rheum rhabarbarum* L. (Polygonaceae), known for its capacity to produce very large leaves in high light environments and small leaves under low light.

MATERIALS AND METHODS

Plant material

Three potted individuals of *R. rhabarbarum* cv. Victoria were grown for the duration of the experiment at either high or low light intensity

from dormant, 5-mo-old rhizomes. Plants were grown in a controlled glasshouse under night/day temperatures of 16/22°C, respectively, and natural photoperiod of ca. 12 h. All plants were watered once per day and received liquid nutrients once per week. Plants grown at high light intensity received unobscured natural light, with a maximum photosynthetic photon flux density (PPFD) of ca. 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, while plants grown at low light intensity were grown in the same environment but under a light-reducing shade cloth, receiving a maximum PPFD of ca. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Excluding the first three leaves following the breaking of rhizome dormancy, which may have been initiated under different environmental conditions, all subsequent leaves were tagged on the day of first emergence from ochrea. Plants grown in high light produced the same number of leaves as those in low light during the experimental period. After 1 month of growth, with all emerged leaves tagged, leaves were harvested so that the collection contained leaves that spanned the full developmental range from initial emergence from ochrea to fully expanded leaves under each light condition. All leaves were immediately stored at -20°C until later processing for anatomy. The age range was defined based on a preliminary experiment carried out with *R. rhabarbarum*, which demonstrated that leaves take 4 weeks to fully expand under both light conditions (Appendix S1, see Supplemental Data with this article). The first day of leaf expansion was considered to be the first day of visible leaf emergence from the ochrea (Appendix S2), but it should be noted that vein and stomatal initiation began before that stage.

Leaf area and anatomical traits during leaf expansion

Leaves were characterized in terms of leaf area and anatomical traits. The densities of minor veins, stomata, epidermal cells, and stomatal primordia were quantified for all leaves in the same cleared and stained leaf segments. Leaf segments for analysis were selected at random from the leaf, taking care to avoid major veins. Extensive work has shown that minor vein density and stomatal density are homogenous across leaves, even in large-leaved species (Fiorin et al., 2016). Meristemoid, guard mother cell, and young guard cells were considered as stomatal primordia (Appendix S3) (Pillitteri et al., 2007). One ca. 200 mm² section was taken from the middle of the lamina of each leaf, avoiding first- to fourth-order veins. For a definition of major vein orders, see section “Major vein density of fully expanded leaves at low and high light” below. The leaf sections were placed in commercial household bleach (8.25% sodium hypochlorite) until clear, rinsed in water at least three times, and stained with 1% w/v aqueous toluidine blue. Five fields of view (FOV) at 10 \times magnification and five FOV at 20 \times magnification were photographed from each leaf section using an Axiocam 503 color digital camera (Zeiss, Jena, Germany) mounted on an Axio Imager A2 microscope (Zeiss, Jena, Germany). Minor vein density was measured as the total length of leaf vascular tissue per mm² of leaf area, based on a full FOV at 10 \times magnification. Stomatal density and epidermal cell size were measured in the same leaf section, based on a full FOV at 20 \times magnification; stomatal density was quantified as the number of stomata per mm² of leaf area, and epidermal cell size was calculated as the FOV excluding stomatal area divided by epidermal cell number within the FOV (Salisbury, 1927). Stomatal area was estimated assuming that each stomatal complex was an ellipse of length corresponding to guard cell length and width being the width of the guard cell pair. All measurements were performed using the software ImageJ (National Institutes of Health,

Bethesda, MD, USA). Finally, total minor vein length and total stomatal and epidermal cell number were calculated by multiplying their density by leaf area for each leaf, which was scanned after excising through undulations to flatten completely with no leaf overlap on a flatbed scanner.

Dilution models during leaf expansion

We compared modelled relationships that assume vein and stomatal density dilution occurred due to passive increases in cell size during leaf expansion starting at day 5 of leaf emergence from the ochrea with the observed relationships of vein and stomatal densities under different light conditions. Day 5 was chosen as the starting point for the model to allow for a period of cell differentiation before passive expansion. The leaf growth rate for each light condition was used to determine the rate of dilution during expansion, using initial leaf area and either initial vein or stomatal density, considering no new vein or stomatal differentiation. The mean vein and stomatal dilution based on passive cell expansion were calculated using leaf growth rate at either high or low light intensity and initial values for mean leaf area, total vein length or total stomatal number, respectively. For that, the following equations were used:

$$\text{Minor vein density at day } t = \frac{\text{Initial total minor vein length}}{\text{Initial leaf area} + (t \times \text{Leaf growth rate})}$$

and

$$\text{Stomatal density at day } t = \frac{\text{Initial total stomatal number}}{\text{Initial leaf area} + (t \times \text{Leaf growth rate})}.$$

Initial leaf area, total vein length, and total stomatal number were assumed to be equivalent to the mean measurements of leaves at day 5, as we assumed 5 d was sufficient time to initiate all veins and stomata after which passive dilution occurred. Leaf growth rate was also obtained using data of leaves from 5 to 28 d following emergence from the ochrea.

Major vein density of fully expanded leaves at low and high light

The density of major veins (i.e., first- to fourth-order veins) was analyzed from images of intact leaves. Vein diameter was not used to categorize vein orders, as diameters from the same vein orders varied between sun and shade leaves, instead we categorized vein orders by branching pattern (Sack et al., 2012; Wen et al., 2018). Leaves of *R. rhubarbarum* are palmately veined (Niinemets et al., 2007), with around five first-order veins branching from the petiole. The second-order veins were defined as those that branched from the first-order veins, and the third- and fourth-order veins were defined as those branched from the second- and third-order veins, respectively (Hickey, 1973). The density of major veins was accessed for three fully expanded leaves (28 d following emergence from ochrea) formed at either low or high light.

Statistical analyses

Data were collected from three plants grown at high light and three grown at low light. The nonlinear associations between the

morphophysiological features were analyzed by generalized additive models using the gam function in the mgcv package (Wood and Wood, 2015) in R (R Core Team, 2014), assuming a normal distribution and with the recommended default value chains smoothing. The significance of the models was tested by ANOVA, and similarities between the slopes for the two light conditions were tested by ANCOVA. The linear relationship between the major vein density and leaf area was also tested by ANOVA.

RESULTS

The increase in leaf area in leaves expanded in high light was 6-fold greater than leaves expanded at low light (Fig. 1). Leaves of *R. rhubarbarum* underwent a rapid expansion phase until the 20th day following emergence from ochrea when leaf expansion ceased

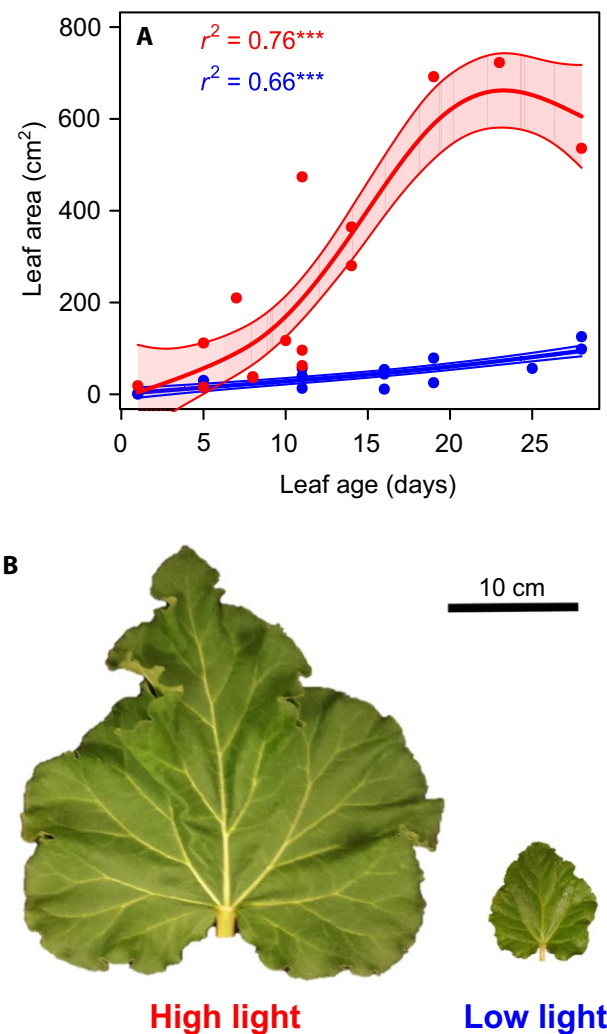


FIGURE 1. (A) Leaf area during leaf expansion in *Rheum rhubarbarum* plants grown under either high (red symbols) or low light intensity (blue symbols). Generalized additive model curves; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, the banding on either side of the model represents the 95% confidence interval. (B) Representative image of 28-d-old leaves fully expanded under either high or low light. Day 1 corresponds to the first day a leaf visibly emerged from ochrea.

abruptly (Fig. 1; $P < 0.001$; Appendix S1). Despite considerable differences in leaf area between high- and low-light-expanding leaves, minor vein density and stomatal density showed similar patterns of decline with age, so that, at any age leaves expanding under both light conditions had similar values (Fig. 2A, B). The patterns for stomatal density and minor vein density with respect to age were subtly different ($P < 0.001$), with a continuous decline in stomatal density starting at the 5th day (Fig. 2B), but a more rapid initial decline (over the first 10 d following leaf emergence from ochrea) in minor vein density (Fig. 2A). The relationship between minor vein density and stomatal density in leaves expanding under low light was slightly steeper from that in high-light leaves ($P < 0.001$; Fig. 2C). Although both low- and high-light-expanding leaves had a higher number of stomata per minor vein length at the beginning compared to the end of leaf expansion, high-light-expanding leaves had more stomata per unit of minor vein than did low-light-expanding leaves at full expansion (Fig. 2C).

Epidermal cell size increased in a similar way during the expansion of both high- and low-light-expanding leaves, with a single regression explaining epidermal cell growth under both light conditions (Fig. 3). For both high- and low-light-expanding leaves, minor vein density declined in response to initial increases in the epidermal cell size, and then it was maintained despite continuous increases in epidermal cell size ($P < 0.001$; Fig. 3). Stomatal density, however, was high and changed little as leaves began to expand, but declined rapidly when epidermal cell size measurably increased ($P < 0.001$; Fig. 3).

The total number of epidermal cells was constant during leaf expansion in leaves grown under both high and low light intensities, with little differentiation of new cells as leaves expanded ($P > 0.05$; Fig. 4A). In contrast, total minor vein length increased as leaves continuously expanded in both light conditions, yet at different rates ($P < 0.01$ for high light and $P < 0.05$ for low light; Fig. 4B). While low-light-expanded leaves maintained a constant number of stomata during leaf expansion, the total number of stomata increased considerably in high-light-developed leaves ($P < 0.05$ for high light and $P > 0.05$ for low light; Fig. 4C). During the first 10 d, a higher density of stomatal primordia was found in leaves grown in high light compared with leaves grown in low light (Appendix S4). Stomatal primordia in high-light-developed leaves diminished in density until the 10th day of leaf emergence from ochrea and after that were no longer observed (Appendices S3, S4).

Observed data for vein and stomatal density during leaf expansion at low light was very similar to the predicted relationships between either vein or stomatal density and leaf age, which assumed dilution in density solely due to increases in leaf area and not differentiation (Fig. 5). However, observed minor vein density was slightly higher than the modelled minor vein density through time, suggesting some vein development as leaves expanded in low light. In high-light-expanded leaves, the observed data of vein and stomatal density changes as leaves expand could not be explained by passive dilution through epidermal cell expansion alone, implying that new veins and stomata were initiated as leaves expanded at high light intensity (Fig. 5). The density of major veins was highly correlated with leaf area for fully expanded leaves grown at both light conditions, large high-light-expanded leaves had a lower major-vein density (0.49 ± 0.01) compared to the smaller leaves that expanded under low light (0.107 ± 0.04) (Appendix S5).

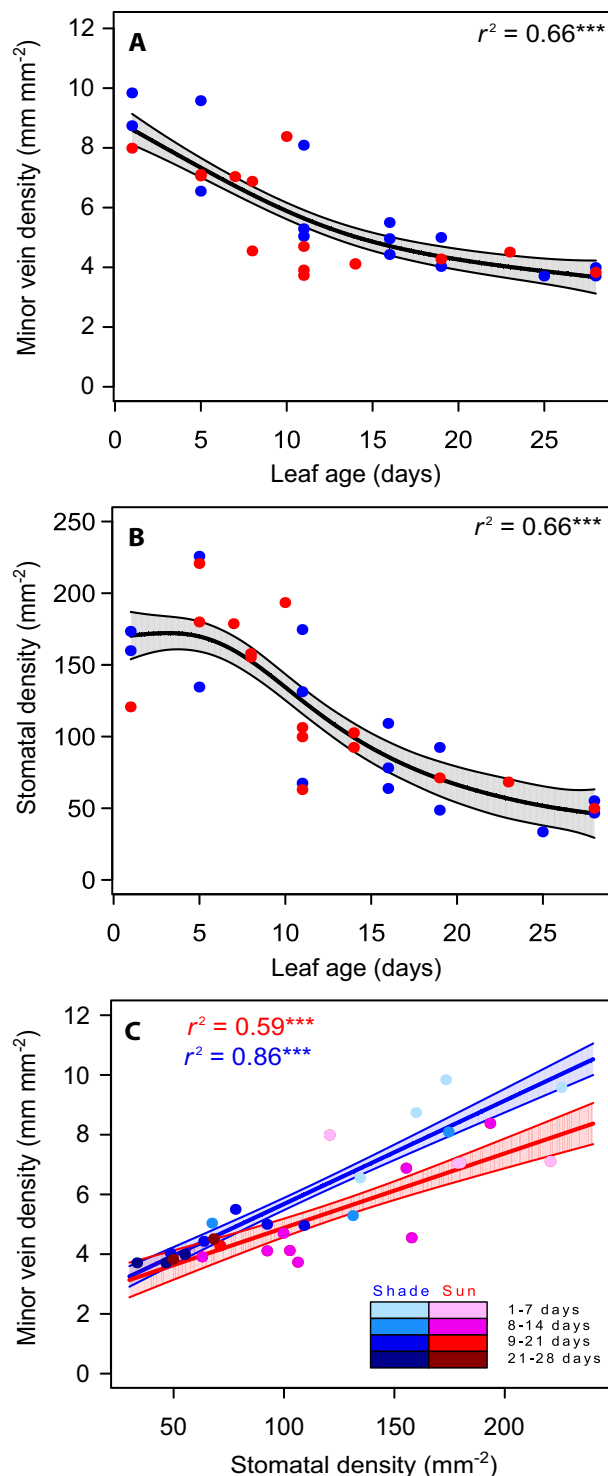
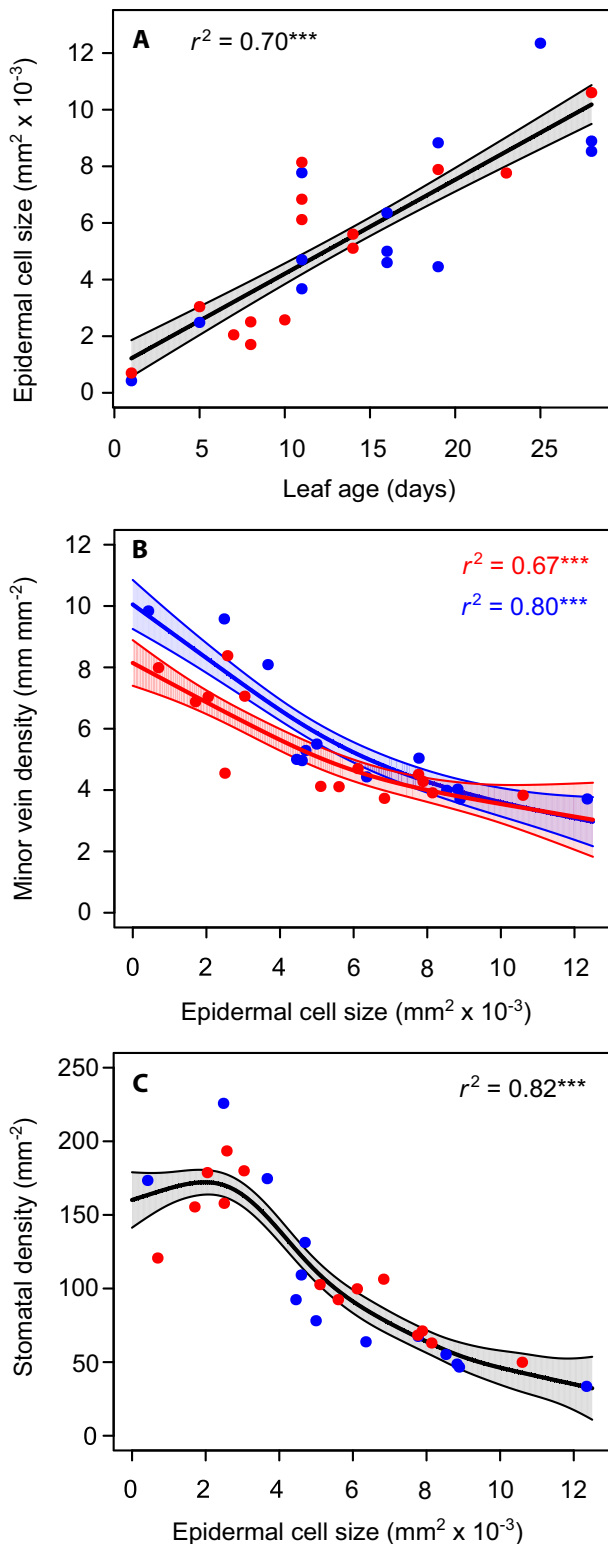


FIGURE 2. (A) Minor vein and (B) stomatal density during leaf expansion and (C) relationship between minor vein density and stomatal density during leaf expansion in *Rheum rhabarbarum* plants grown under either high (red symbols) or low light intensity (blue symbols). In (C) different color shades represent different ranges of leaf age. Generalized additive model curves: test of significant relationship between the variables: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, the banding on either side of the model represents the 95% confidence interval. Day 1 corresponds to the first day a leaf visibly emerged from ochrea.



DISCUSSION

This study demonstrates that in a herbaceous species with a large degree of plasticity in leaf area, vein and stomatal densities are coordinated by the regulation of minor vein and stomatal

FIGURE 3. (A) Epidermal cell size during leaf expansion and (B) the relationship between minor vein and (C) stomatal density with epidermal cell size in *Rheum rhabarbarum* plants grown under either high (red symbols) or low light intensity (blue symbols). Generalized additive model curves: test of significant relationship between the variables: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, the banding on either side of the model represents the 95% confidence interval. Day 1 corresponds to the first day a leaf visibly emerged from ochrea.

differentiation during leaf expansion. This regulatory mechanism contrasts with many other herbaceous and woody plants that regulate stomatal and vein densities passively by changing cell size and not via differentiation (Carins Murphy et al., 2012, 2016). The addition of extra veins and stomata provides a mechanism that allows the shade-intolerant geophyte, *R. rhabarbarum*, to achieve large leaf area with high vein and stomatal densities in high-light environments. This mechanism may be especially important for the many herbaceous geophyte species in which leaves are the dominant aboveground part of the plant, and the expansion of large leaves minimizes competition with other shade-intolerant competitors. In species where a strong selective pressure is imposed for large leaves in high-light environments, the active differentiation of veins and stomata would allow plants to maximize leaf area without decreasing leaf gas-exchange capacity or increasing susceptibility to damage by desiccation.

Continuous development of minor veins and prolonged initiation of stomata during ongoing leaf expansion at high light

Major vein density was highly correlated with leaf area in both high- and low-light-expanded leaves, decreasing as leaf area increased (Appendix S5). Declines in major vein density with increasing leaf area are expected given that these veins are formed in limited quantities in the primordial leaf (Sack et al., 2012), and for this reason, final major vein density exhibits a strong correlation with leaf area (Sack et al., 2012). Unlike declines in major vein density driven by increases in leaf area, the same stomatal and minor vein densities were observed in low- and high-light-expanded leaves of *R. rhabarbarum* despite 6-fold differences in leaf area (Fig. 2A). This similarity in vein density across leaf sizes in this species can be explained by the active differentiation of new minor veins and stomata during leaf expansion in large leaves grown under high light (Fig. 4). Light quantity has been long demonstrated to induce stomatal development (Schoch et al., 1980; Lee et al., 2017), and here we also observe a prolonged initiation of veins under higher light intensity.

The window for active differentiation for minor veins and stomata at high-light intensity seemed to be subtly different. Vein differentiation occurred over the whole period of leaf expansion (Fig. 4B). This continuous development of minor veins during leaf expansion was predicted in *Arabidopsis thaliana* (Sack et al., 2012). In contrast, the initiation of large numbers of new stomata (and epidermal cells) occurred only until the 10th day following leaf emergence from ochrea, corresponding with the age of leaves in which stomatal primordia were present and leaves reached a maximum rate of expansion (Appendix S4). A short period of stomatal initiation has been observed in the small-leaved-species *Arabidopsis thaliana* (Nadeau and Sack, 2002). The continuous development of

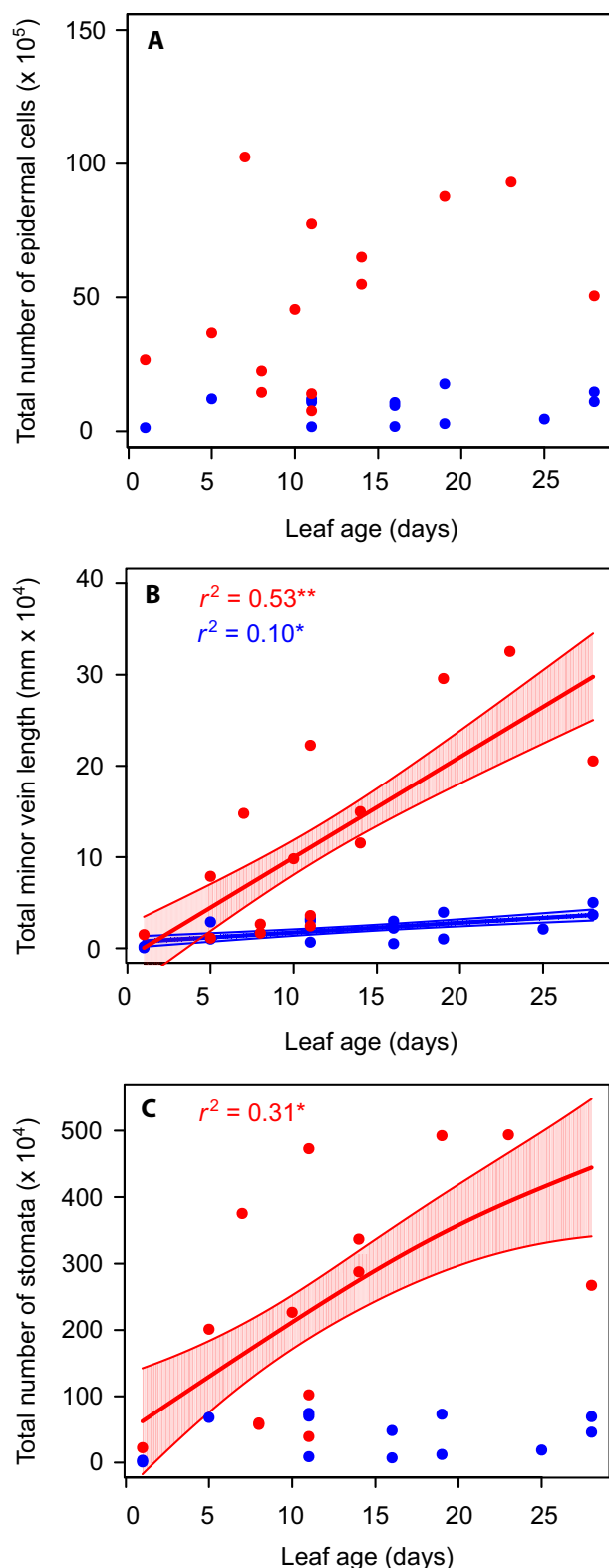


FIGURE 4. (A) Total number of epidermal cells, (B) minor vein length, and (C) number of stomata in leaves of *Rheum rhabarbarum* plants during leaf expansion grown under either high (red symbols) or low light intensity (blue symbols). Generalized additive model curves: test of significant relationship between the variables: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, the banding on either side of the model represents the 95% confidence interval. Day 1 corresponds to the first day a leaf visibly emerged from ochrea.

Vein and stomatal densities are controlled by independent mechanisms

Our observations in *R. rhabarbarum* add to a growing body of evidence suggesting that highly independent regulatory mechanisms control the development of veins and stomata, despite the strong developmental coordination in the densities of these two key anatomical features (Carins Murphy et al., 2017; McAdam et al., 2017). The initiation of veins and stomata has been extensively described yet no apparent cross-talk between these two coordinated features of plant leaves has been suggested. The development of a leaf vein network has been linked to auxin biosynthesis and signaling, with auxin-mutants presenting reduced vein densities (Tobena-Santamaria et al., 2002; Verna et al., 2015; McAdam et al., 2017). These mutations have been observed to result in considerable impaired vein formation independently of any changes in stomatal density or anatomy (McAdam et al., 2017). Stomatal development, involves several well-described stages, including asymmetric and symmetric divisions from meristemoids into a pair of guard cells (Pillitteri et al., 2007). These stages have been demonstrated to be spatially and temporally regulated by the key genetic regulators *SPEECHLESS* (*SPCH*), *MUTE*, and *FAMA* (Pillitteri et al., 2007) as well as inducer of CBF expression (ICE) transcription factors (Kanaoka et al., 2008). Like angiosperms (Schoch et al., 1980; Lee et al., 2017), fern stomatal initiation has been found to be augmented by high light intensity (Carins Murphy et al., 2017) and, interestingly, this increased stomatal differentiation at high light in ferns was independent of modifications in the leaf vein network (Carins Murphy et al., 2017).

The temporally independent differentiation of veins and stomata, allows the deviation in the proportional relationship between the densities of these two features to occur during leaf expansion (Fig. 2C). The higher production of veins than stomata during the final stages of leaf expansion in *R. rhabarbarum* resulted in the highest vein length per number of stomata being observed when leaves had fully expanded. The lower vein to stomatal ratio in young, incompletely expanded leaves of this species suggests undersupply of water to the stomata in these leaves places a considerable hydraulic limitation on stomatal opening.

CONCLUSIONS

We demonstrate here that, unlike most shade-tolerant species, the shade-intolerant species *Rheum rhabarbarum* can adjust the initiation of stomata and minor veins under high-light environments, facilitating the production of larger leaves uncoupled from lower vein and stomatal densities. Such a mechanism is likely to be a critical determinant of leaf anatomy in a wide range of shade-intolerant geophytes, in which large leaves provide a strong competitive advantage in high-light environments. Given strong evidence for independent

minor veins yet shorter period of stomatal initiation suggests that final leaf area may be developmentally determined from an early stage of leaf expansion and closely related to the degree of stomatal initiation during the first 10 days of leaf expansion.

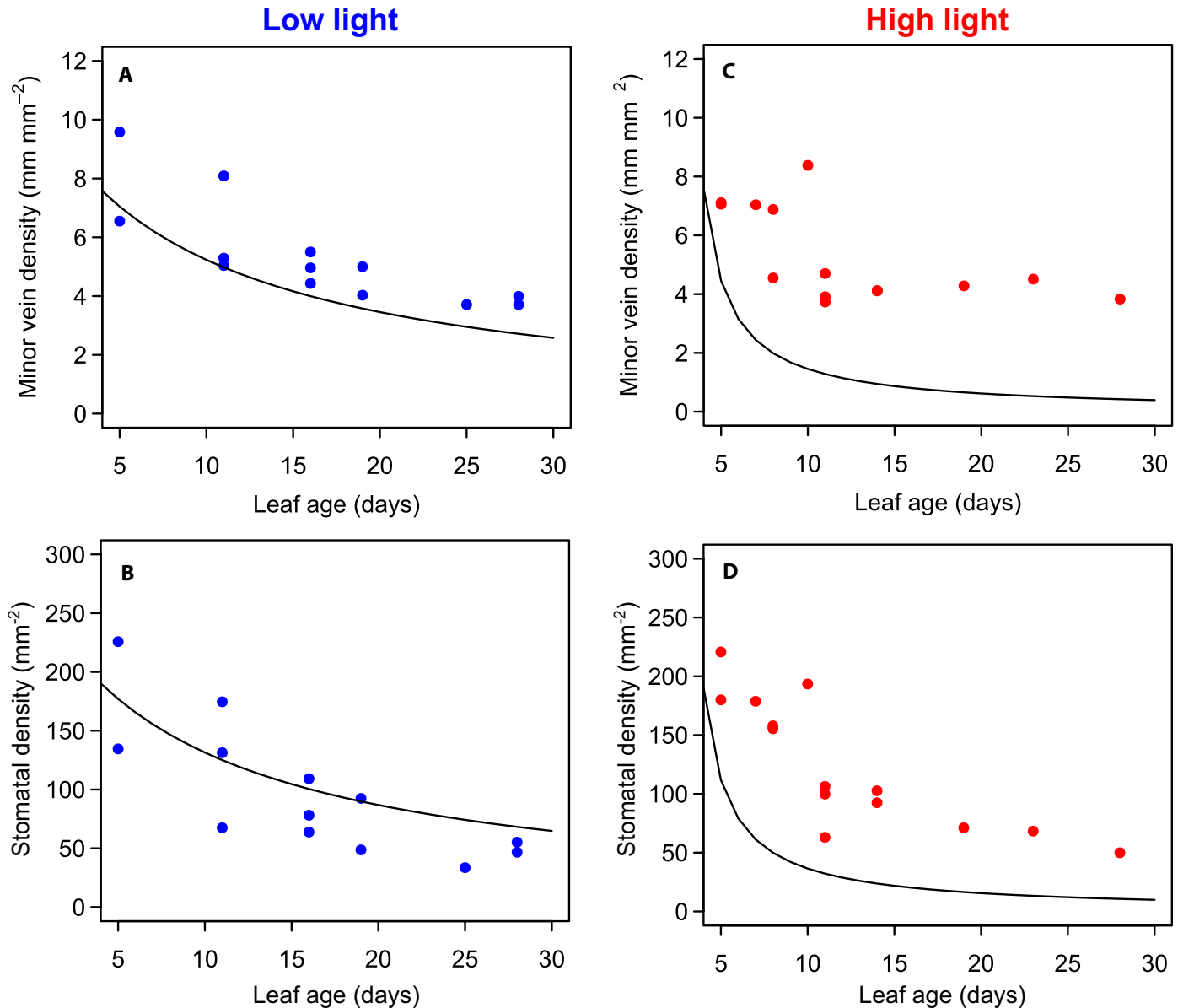


FIGURE 5. Modelled (lines) and observed (symbols) (A, C) minor vein density and (B, D) stomatal density during leaf expansion in *Rheum rhabarbarum* plants grown under either high (red symbols) or low light intensity (blue symbols). Observed data are taken from Fig 2. Modelled data, presented as the black lines, assume vein and stomatal density dilution due to increases in leaf area in the absence of new vein and stomatal differentiation during leaf expansion (see the Materials and Methods for details).

controls on the initiation of veins and stomata, this adjustment must involve some form of developmental control that is both currently unknown and which is not active in shade-tolerant species.

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AUTHOR CONTRIBUTIONS

S.A.M.M. and G.J.J. designed the study; A.A.C. and J.M.R. carried out the experiments; A.A.C., S.A.M.M. and G.J.J. analyzed the data; A.A.C., S.A.M.M. and G.J.J. wrote and revised the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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